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Obtaining a New Gene-Cell Construct Based on Transduced Olfactory Ensheathing Cells for the Treatment of Spinal Cord Injuries

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We developed a viral vector Ad5/35-CAG-mBDNF expressing the mature form of BDNF (mBDNF). On the basis of olfactory ensheathing cells transduced with this adenovector, a new gene-cell construct was obtained. In experiments *in vitro*, high viability of the transduced olfactory ensheathing cells and enhanced secretion of BDNF by these cells were observed. It is possible that a new gene-cell construct will significantly increase the regenerative effects of transplanted olfactory ensheathing cells.

Key Words: *gene-cell therapy; spinal cord injury; olfactory ensheathing cells; brain-derived neurotrophic factor*

Spinal cord injuries lead to complete or partial denervation of the limbs and internal organs as a result of death of neurons and glial cells, and to disability of patients [14]. Regenerative processes in the nervous tissue of the spinal cord after injury are limited by inflammation, neuron death, formation of glial scars and cysts, as well as imbalance of neurotrophic factors against the background of injury [3]. The most important role of neurotrophic factors (BDNF, NGF, NT-3, and NT-4/5) is related to their ability to exert neuroprotective effects and promote neuronal regeneration [9]. The stimulating and neuroprotective effects brain-derived neurotrophic factor (BDNF), the key neurotrophic factor, have been shown in various structures of the spinal cord [11]. However, the expression of neurotrophic factors in chronic spinal cord injury is very low [10]. Thus, an urgent task is to restore their balance by targeted stimulation of production or exogenous delivery.

Great hopes are associated with gene therapy based on various viral vectors that can easily cross the cell membranes and deliver genetic material to the nucleus, where it is transcribed into mRNA [17]. As a result, the infected cell acquires the ability to constitutively or transiently express the transgene. The use of viral vectors for expression has advantages over the introduction of recombinant neurotrophic factors into the damaged area, which, when used in free form, have a half-life of only a few minutes [2]. However, gene therapy using viral vectors has its limitations. First, for the safe clinical use of a viral vector, a wild-type modification of the virus is required to block viral reproduction in cells [1]. Secondly, there is a problem with the efficiency of virus delivery, which consists in the fact that in some cases the virus carrying the transgene can be inactivated by antibodies after introduction [13]. Gene-cell constructs are used to solve this problem [4]. With this approach, the cells will not only limit the contact of the viral vector with

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the immune system, but they themselves will have regenerative effects.

Olfactory ensheathing cells (OEC) obtained from the nasal olfactory mucosa can be the most optimal basis for a gene-cell construct. The efficiency of OEC into the spinal cord in various types of injury has been demonstrated in studies [5,7]. We have also shown that the transplantation of these cells into post-traumatic cysts significantly improves the motor activity of the hind limbs of rats and reduces the size of the cysts [19]. OEC secrete a number of neurotrophic factors, including BDNF [20]. It is possible that the transduction of these cells, aimed at additional expression of BDNF, will significantly increase the regenerative effects of transplanted OEC.

The aim of this study was to obtain a new gene-cell construct based on transduced OEC expressing BDNF for the treatment of spinal cord injuries.

MATERIALS AND METHODS

Reagents of analytical purity Sigma-Aldrich, Thermo Fisher Scientific, and HyClone were used in the work. To create an adenoviral vector, a rat cDNA nucleotide sequence encoding the mature form of BDNF (mBDNF) was used. Amplicons with cDNA of mBDNF and firefly luciferase (Fluc) and corresponding restriction sites at the ends were obtained by PCR: HindIII at the 5'-end and BglII at the 3'-end. Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific) was used for PCR, Bdnf(NM_001270630) Rat Untagged Clone RN216484 (Origene) or pLA-NP-luc-H4-puro-1 plasmid was used as a template, and primers with the sequence Fwd_sp-mBDNF_HindIII: GAGCAAGCTTGCCACCATGCGGATCCTGCTGCTGACCATGGTGATCTCCTACTTCGGCTGCATGAAGGCCCACTCCGACCCCGCC and Rev_mBDNF_BglII: TGAGCAGATCTCTATCTTCCCCTTTTAATGGTCAGTGTACA. The resulting amplicon was inserted into the pShuttle-CAG plasmid (#13427, AddGene) under the control of a strong synthetic CAG promoter by the restriction ligation method.

Recombinant adenoviruses were generated by homologous RED/ET recombination (induced by arabinose) using a selection and counterselection system based on the *rpsL* gene encoding the S12 ribosomal protein, which is the target of streptomycin (Counter Selection BAC Modification Kit; Gene Bridges). For recombination, we used a previously constructed plasmid containing the complete genome of the human adenovirus serotype 5 chimeric for the fiber protein — Ad5/35-E1Adelta24, in which the Ad5 fiber protein is replaced by $^{2}/_{3}$ by the adenovirus fiber protein serotype 35, as well as a deletion of 24 nucleotides in the E1A locus [fifteen]. For reverse recombination, a pShuttle-CAG-mBDNF/Fluc plasmid fragment obtained by restriction of the plasmid with PacI and SacI was used. As a result of homologous recombination, a vector was obtained containing a transgene under the regulation of the promoter, inserted instead of the E1 locus of adenovirus.

HEK293 cells were transfected with linearized adenovectors using Lipofectamine 3000 (Invitrogen). Virus amplification was performed on 911 cells. Virus purification, storage, and titration were performed as described previously [15]. To determine the titer of infectious particles (infectious units, IFU), recombinant adenoviruses were titrated on 911 cells using the Adeno-X Rapid Titer Kit (Takara Bio) according to the manufacturer's instructions.

OEC were obtained from the olfactory mucosa of Wistar rats according to the protocols developed in the Department of Fundamental and Applied Neurobiology of the V. P. Serbsky National Medical Research Center of Psychiatry and Narcology [18]. Passage 3-4 cells were used in further *in vitro* experiments.

The transduction potential of previously constructed conditionally competent adenoviruses with different tropisms: Ad5/35-E1B-p2A-eGFP and Ad5RGD-E1B-p2A-eGFP containing green fluorescent protein (GFP) was assessed. The fiber protein of adenovector Ad5/35 is substituted by $^{2}/_{3}$ for serotype 35 [8], which ensures interaction with CD46 receptors. Ad5RGD contains a genetic modification, which is the incorporation of the arginine-glycine-aspartate (RGD) motif of the αV integrin-binding peptide RGD-4C (CDCRGDCFC) into the HI-loop of the fiber protrusion domain [15]. To this end, OEC of the olfactory mucosa of rats (100,000 cells per well) were seeded in a 24-well plate and infected in suspension with adenovectors Ad5RGD-E1B-p2A-GFP and Ad5/35-E1B-p2A-GFP with a multiplicity of infection (MOI) of 500 infectious particles per cell. A comparison of the transduction potential of adenovectors with Ad5RGD and Ad5/35 tropism was performed 24 h after cell infection using a Leica 3000 microscope with the possibility of fluorescent photography to assess the GFP luminescence intensity.

To assess the survival of OEC infected with the obtained adenovirus particles, the cells were seeded in a 96-well plate (5000 per well; 80 μl of medium with 10% fetal bovine serum) and infected with adenovectors in suspension (20 μl of viral inoculum) with MOI 10, 50, 100, 500 infectious particles per cell. After 3 days, the supernatant was aspirated and 100 μl of complete medium with 10% resazurin reagent (Alamar blue; Thermo Scientific) was added to each well. After 4 h of incubation, fluorescence was measured on a Perkin Elmer multiplate reader with excitation and emission wavelengths set according to the manufacturer's recommendations.

mBDNF expression and secretion by OEC was assessed by ELISA (Total BDNF Quantikine ELISA Kit; R&D Systems) at 2, 4, 6, and 8 h. OEC were infected in suspension at an MOI of 50 infectious particles per cell.

The results were statistically processed using the GraphPad Prism 8.3 software (GraphPad Software, Inc.). To assess the significance of differences, the nonparametric Mann–Whitney test was used at $p<0.05$. Data are presented as $M \pm SD$.

RESULTS

When evaluating the ability of OEC to transduce fiber-protein chimeric adenoviruses Ad5/35-E1B-p2A-GFP

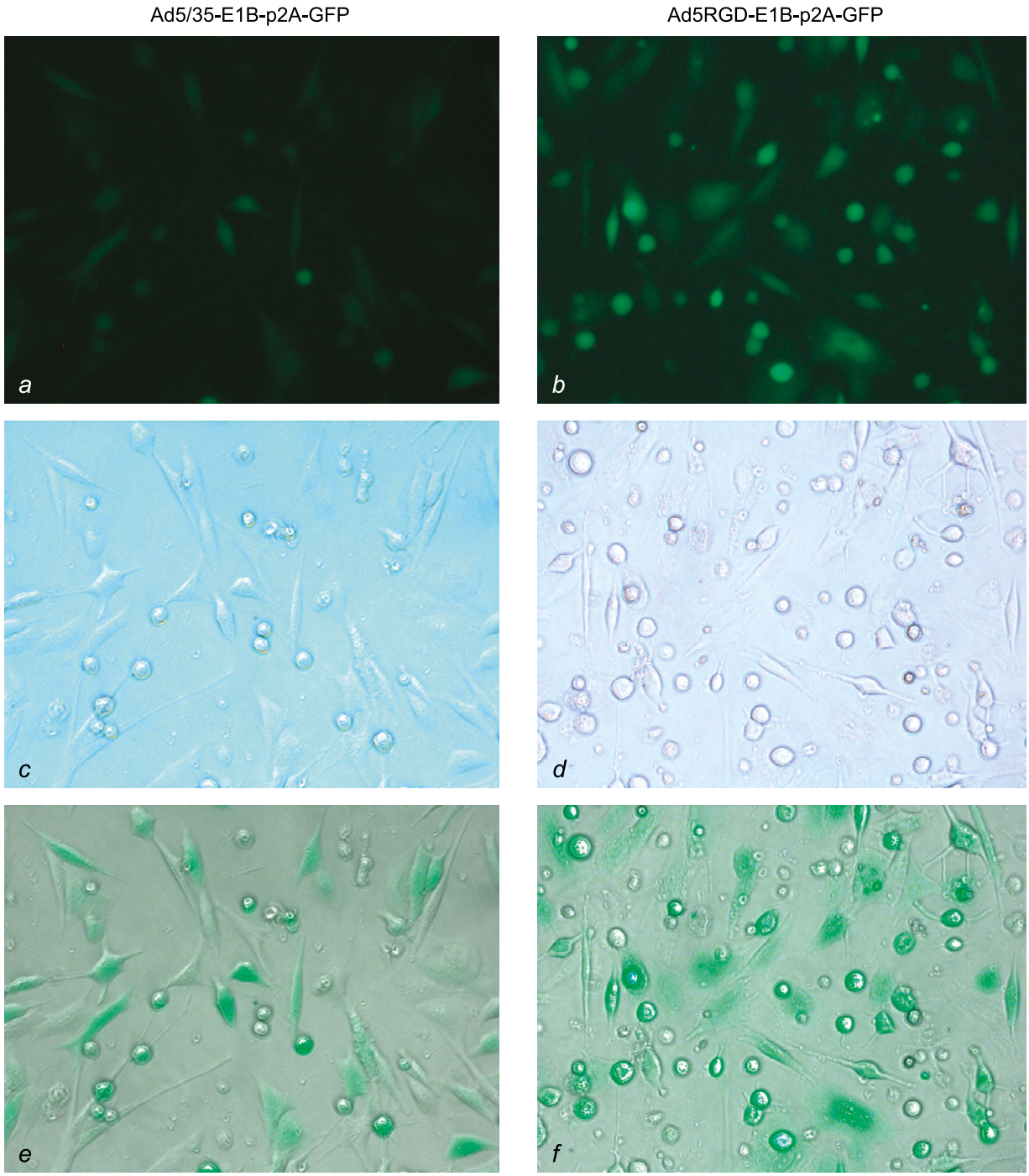

Fig. 1. Transduction potential of adenovectors with Ad5RGD and Ad5/35 tropism estimated by GFP luminescence intensity in 24 h after cell infection with conditionally replication-competent adenovectors Ad5/35-E1B-p2A-GFP and Ad5RGD-E1B-p2A-GFP with MOI=500 viral particles per cell, ×100.

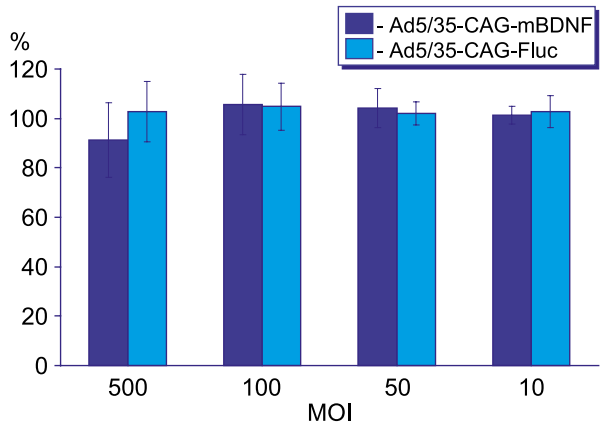

Fig. 2. Viability of rat OEC infected with adenovectors. Cell viability was assessed on day 3 using a resazurin solution (Alamar blue). Uninfected cells are taken as 100%.

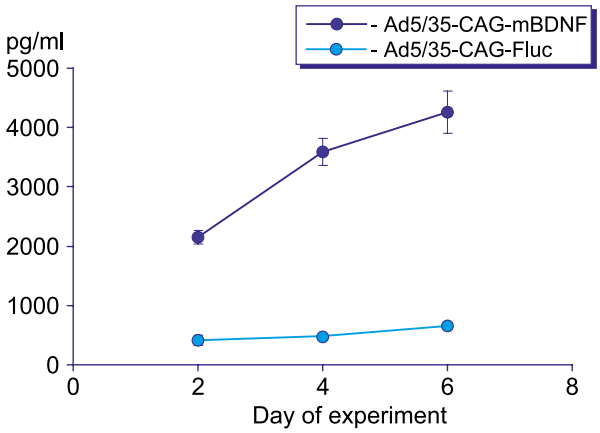

Fig. 3. BDNF secretion by OEC according to ELISA data in 2,4, 6, and 8 h after transduction.

and Ad5RGD-E1B-p2A-GFP by the intensity of OEC luminescence, it was shown that both of these adenovectors have a high transduction potential at MOI=500 (Fig. 1). At the same time, Ad5RGD-E1B-p2A-GFP ensured higher transduction and caused more pronounced cytotoxicity.

When creating vectors, viral carriers are most often used: adenoviruses, adeno-associated viruses, lentiviruses, and herpes simplex virus [17]. We used adenoviruses, as they are the most studied and have certain advantages. Adenovirus vectors effectively transfer genes to both dividing and nondividing cells, do not integrate into the genome, provide high titers of the recombinant virus and a high level of expression of the introduced genes [12]. In addition, they are easy to modify to prevent replication; after a sufficient amount of virus has been produced, it is cleaned according to standard protocols, since cleaning from other viruses is required. We have previously demonstrated the therapeutic effect of human OEC transduced with chimeric adenovirus Ad5/35 with BDNF using an animal model of spinal cord cysts [16]. However, it is known that rodent cells, including rat OEC, are less sensitive to human adenovirus than human cells [15]. Thus, an important task was to determine the ability of Ad5-based chimeric viruses to infect rat cells.

In this work, we studied two chimeric adenoviral constructs at once, which, as shown by various researchers, transduce various human cells with high efficiency [8,15]. In the chimeric Ad5/35, the fiber protein is replaced by serotype 35 by $^{2}/_{3}$, which ensures interaction with the CD46 receptor, which is expressed at a high level on the surface of human cells [8]. The tropism of this virus to human cells of different types has been shown [6,8]. At the same time, we did not find data on the efficiency of transduction of rat cells in the available literature. We found that this virus also successfully infects rodent cells, however, its viral particles per cell require several times more than for transduction of human cells.

Thus, we have designed and obtained a replication-incompetent adenovector based on the Ad5/35 chimera encoding the mature form of BDNF.

The viability of rat OEC transduced with the chimeric Ad5/35-CAG-mBDNF virus and infected with the control Ad5/35-CAG-Fluc virus was compared relative to the survival of OEC that were not transduced. The best survival of Ad5/35-CAG-mBDNF transduced cells was observed at MOI 50 and 100 (Fig. 2) and was 104.2±7.9 and 105.6±12.1%, respectively, relative to the survival of non-transduced cells. The viability of rat OEC transduced with Ad5/35-CAG-mBDNF at these MOI was comparable to that of OEC transduced with the control adenovector. MOI=50 was chosen for further experiments on the study of rat OEC transduced with Ad5/35-CAG-mBDNF.

The obtained chimeric viral vector Ad5/35-CAG-mBDNF had high transduction potential, significantly increased (by ~7 times) the expression of BDNF by OEC during the entire observation period in comparison with that of OEC infected with the control plasmid Ad5/35-CAG- Fluc (Fig. 3). *In vitro* data on BDNF overexpression in transduced rat OEC suggest that these cells will express it at a high level after transplantation into the spinal cord compared to non-transduced cells.

The chimeric viral vector Ad5/35-CAG-mBDNF was developed by us specifically for the transduction of OEC of the olfactory mucosa of rats. The creation of an optimal gene-cell construct for the treatment of traumatic and neurodegenerative diseases of the CNS is a challenging task in molecular and cell biology, but represents a promising area of research. The development of such a gene-cell preparation is extremely important for further clinical studies of patients with post-traumatic spinal cord cysts.

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